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Discovery of a new minnow species in the Black and Marmara Sea basins, Türkiye

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Abstract

Using morphometric, meristic, molecular, and qualitative characters, it confirmed a specific status of a clade discovered. *Phoxinus kottelati*, a new species, is distributed in the southwestern Black and Marmara Sea drainages. It is distinguished by having the scales of the breast scaled but separated by unscaled areas anteriorly, a color pattern in the spawning period, and a blue spot between the eye and the mouth in males. Molecular data also indicate that *Phoxinus kottelati* is characterized by a 2.1% sequence divergence in the mitochondrial DNA cytochrome b (cyt *b*) gene from its closest relative *P. strandjae*, 2.8% from *P. abanticus*, and 3.0% from *P. radeki*.

Key Words

Cyt *b*, freshwater fish, *Phoxinus*, taxonomy

Introduction

Freshwater ecosystems harbor a high level of biodiversity (Haase et al. 2023). However, as smaller aquatic ecosystems, they face greater pressure from pollution, habitat degradation, climate change, invasive species pressure, etc. (Vörösmarty et al. 2010; Dudgeon 2010). Therefore, understanding the biodiversity within these systems is critical for both conservation efforts and advancing our knowledge of freshwater ecology. These findings highlight the possibility of further cryptic variety in the genus, especially in freshwater habitats that are not well understood (Dudgeon et al. 2006).

Phoxinus Rafinesque, 1820, a genus of small freshwater Leuciscid fish (Artaev et al. 2024; Bayçelebi et al. 2024), is represented globally by approximately 26 species (Eschmeyer et al. 2024). Members of this genus are distributed across Europe and Asia, including the Caucasus region and the drainages of the Black, Aegean, and Azov Sea drainages (Kottelat 2007; Vucić et al. 2018; Bogutskaya et al. 2023; Turan et al. 2023; Artaev et al. 2024; Bayçelebi et al. 2024). In Türkiye, only two species of *Phoxinus* had been documented prior to studies: *P. colchicus* Berg, 1910, found in the Çoruh River southeastern Black Sea basin, and *P. strandjae* Drensky, 1926, found in coastal rivers southwest of the Black Sea basin in the Thrace region and Lake Sapanca drainage (Kottelat 2007; Bayçelebi et al. 2015; Saç and Özuluğ 2015). However, research conducted since 2023 has significantly expanded our understanding of *Phoxinus* diversity in Türkiye. Two new species have been described: *P. abanticus* Turan, Bayçelebi, Özuluğ, Gaygusuz & Aksu, 2023, from the Lake Abant drainage, and *P. radeki* Bayçelebi, Turan & Aksu, 2024, from the Ergene River in the Aegean Sea basin.

The aim of this study is to investigate the minnows in the southwestern Black Sea and Marmara Sea basins by using both morphological and genetic data. This study tests whether the newly investigated populations belong to a new species or not. Additionally, this study seeks to underscore that it contributes to the broader understanding of freshwater fish diversity in the regions.

Material and methods

Fish sampling and measurements

Individuals were collected by Samus 1000 pulsed DC electro-fishing equipment. After anesthesia, specimens were

fixed in 5% formaldehyde and stored in 70% ethanol or directly fixed in 96% ethanol (you can see the sampled stations and species in Fig. 1). Methods for counts and measurements follow Kottelat and Freyhof (2007) and for nuptial coloration Denys et al. (2020). Measurements were performed using a dial calliper with a precision of 0.1 mm, following a point-to-point methodology. Standard length (SL) was measured from the tip of the upper lip to the end of the hypural complex. The length of the caudal peduncle was measured from behind the base of the last anal-fin ray to the end of the hypural complex, at mid-height of the caudal-fin base.

In dorsal and anal fins, the last two branched rays articulate on a single pterygiophore were counted as "1½." Fin rays and scales were counted under a stereomicroscope. All body measurements were standardized to each individual's standard length (SL). The collected samples are deposited at the Recep Tayyip Erdogan University Zoology Collection of the Faculty of Fisheries (FFR).

Abbreviations used: **SL:** standard length; **HL:** head length; **SD:** standard deviation.

The animal welfare laws, guidelines, and policies of the Republic of Türkiye approved by the Recep Tayyip Erdogan University Animal Experiments Local Ethics Committee (2014/72) were followed for the care and use of experimental animals.

DNA isolation, amplification, and sequencing

Total DNA from ethanol-preserved tissue of *Phoxinus* specimens was isolated with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The Cytochrome b (Cyt *b*) gene of vertebrate mitochondrial DNA was amplified using the primers "AlbCF (5′-CAACTACAAGAACATGGCAAGCC-3′) and AlbCR (5′-CTTCGGATTACAAGACCGATGC-3′)" described by Bektaş et al. (2019).

The PCR protocol and thermocycler conditions were performed according to Turan et al. (2023). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and both directional sequencing of PCR products was performed with the same primers used for amplification at Macrogen Europe using an ABI PRISM 3730×1 Genetic Analyzer and a BigDye Terminator 3.1 cycle sequencing ready reaction kit (Applied Biosystem).

Molecular data analyses

In this study, *Phoxinus* species distributed in Türkiye were examined, and 25 cyt *b* gene region sequences were generated. The Clustal W algorithm (Thompson et al. 1994) in Bioedit v7.2.5 (Hall 1999) was used to align cyt *b* sequences, and the sequences were submitted to NCBI GenBank with accession numbers PQ699153–PQ699177. The nucleotide substitution model was determined in MEGA 11 (Tamura et al. 2021), and the TN93 + I model (Tamura and Nei 1993) was selected as the best-fit substitution model for the cyt *b* gene. Phylogenetic relationships among species were carried out using

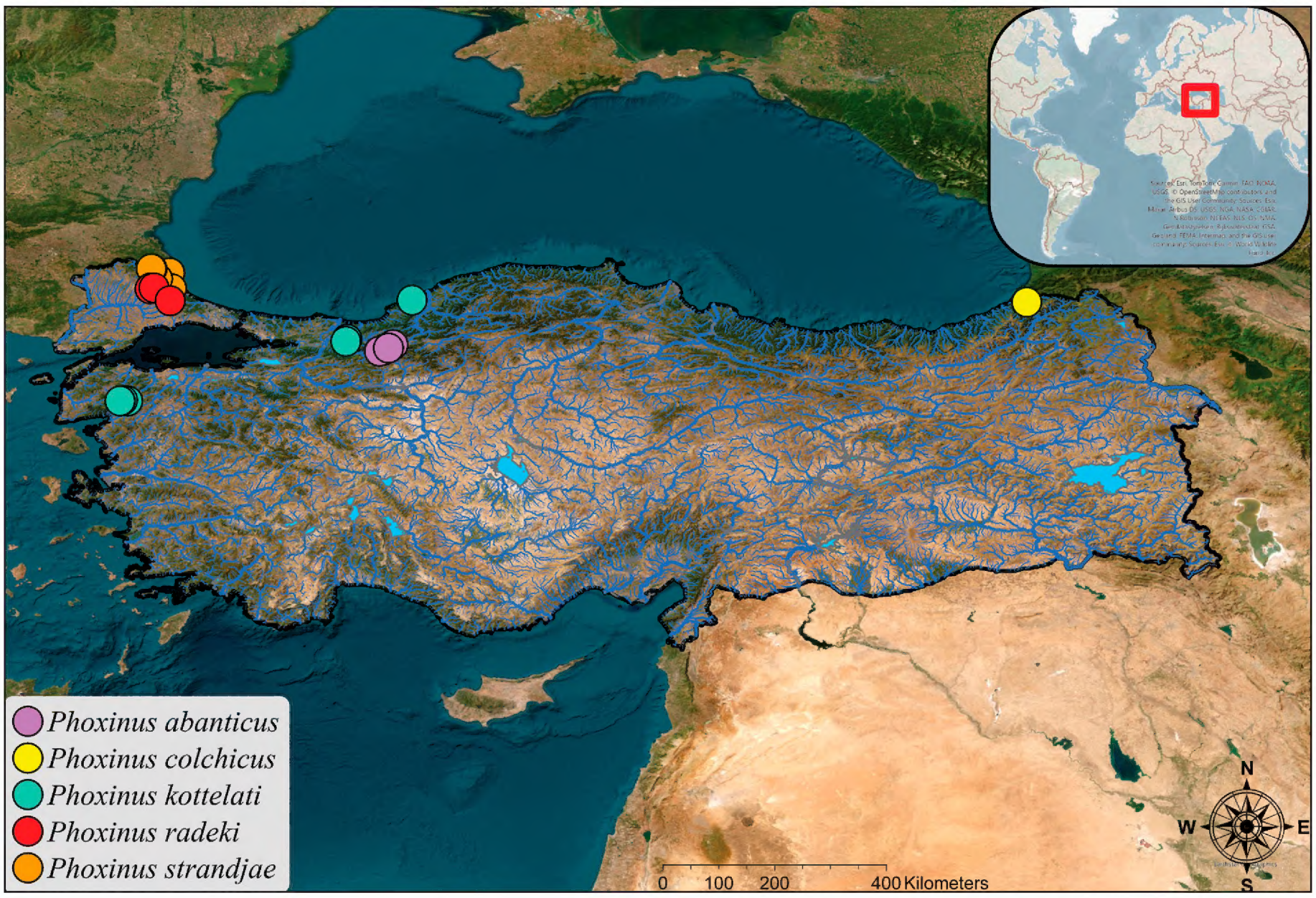

Figure 1. Distribution of *Phoxinus* species in Türkiye.

maximum likelihood (ML) analysis using MEGA 11. Also, MEGA 11 was used to estimate interspecies genetic distance with the p-distance model. The out-group taxon was selected as *Alburnoides fasciatus* (MK860065) for phylogenetic analysis.

Results

***Phoxinus kottelati* sp. nov.**

https://zoobank.org/C51D30F9-3542-47D9-A2B3-8B64DE617B2A

Figs 2–5

Materials examined. ***Holotype.*** FFR 2328, 49 mm SL; Türkiye: Çanakkale prov.: stream Koca about at Kalkım, 39.8144°N, 27.2299°E.

Paratypes. FFR 2310, 34 specimens, 38–68 mm SL, same data as holotype. — FFR 2308, 92 specimens, 33–56 mm SL; Türkiye: Sakarya Province: stream Uludere at Hendek, 40.8068°N, 30.7581°E. — FFR 2315, 5 specimens, 43–53 mm SL; Türkiye: Çanakkale Province: stream Koca, about 3 km southwest of Aşağıçavuşlu village, 39.8269°N, 27.1467°E. — FFR 2316, 14 specimens, 29–70 mm SL; Türkiye: Çanakkale Province: stream Koca at Aşağıçavuşlu village, 39.8120°N, 27.1085°E. — FFR 2318, 20 specimens, 43–65 mm SL; Türkiye: Sakarya Province: stream Uludere at Hendek, 40.7710°N, 30.7384°E. — FFR 2319, 16 specimens, 25–63 mm SL; Türkiye: Çanakkale Province: stream Koca near Aşağıçavuşlu village, 39.8131°N, 27.1061°E. — FFR 2323, 31 specimens, 41–67 mm SL; Türkiye: Zonguldak Province: stream Akgüney, Black Sea basin, 41.4355°N, 31.8041°E.

Genetic material. FFR-DNA-PH 17-18-19-21-22-23-24-25; Türkiye: Çanakkale Province: stream Koca near Kalkım, 39.8144°N, 27.2299°E (GenBank accession numbers: PQ699164–PQ699171). — FFR-DNA-PH 2, 3, 4, 6, 7, 8, 9, 11, 13, 14; Türkiye: Sakarya Province: stream Uludere at Hendek, 40.7710°N, 30.7384°E (GenBank accession numbers: PQ699153–PQ699163). — FFR-DNA-PH 48, 49, 50, 51, 52, 53; Türkiye: Zonguldak Province: stream Akgüney, Black Sea basin, 41.4355°N, 31.8041°E (GenBank accession numbers: PQ699172–PQ699177).

Figure 2. *Phoxinus kottelati*, from top: FFR 2323, 55 mm SL male; 54 mm SL female; stream Akgüney. Photo by: Cüneyt Kaya.

Diagnosis. *Phoxinus kottelati* is distinguished from other *Phoxinus* species in the Black Sea basin and Lake Abant drainage by a combination of characters, none of them unique. *Phoxinus kottelati* is distinguished from *P. abanticus*, *P. colchicus*, and *P. strandjae* by having breast scaled but separated unscaled area anteriorly in males (vs. absent in *P. abanticus*, breast scaled and scaled area connected anteriorly in *P. colchicus*, breast scaled connected or scales not connected anteriorly in *P. strandjae*) (Fig. 6). *P. kottelati* is further distinguished from *P. abanticus*, *P. colchicus*, and *P. strandjae* by having color pattern in spawning period in males. In *P. kottelati*, Z1 greyish with small and irregularly shaped brownish spots (vs. brownish with small irregularly shaped blackish spots in *P. abanticus*, light brown with vertically elongated pale blotches in *P. colchicus*, dark brown in *P. strandjae*), Z2 a light green stripe along the body (vs. disappeared in the front of the body, slightly distinct in the posterior part of the body in *P. abanticus*, indistinct in both posterior and anterior parts of body in *P. colchicus*, distinct in both anterior and posterior parts of body in *P. strandjae*), Z3 and Z4 almost completely the sides of the body are distinctly green (vs. yellowish and only distinct in anterior part of the body in *P. abanticus*, absent in *P. colchicus* and in *P. strandjae*), and Z5 a yellowish line from pectoral to anal fins origin (vs. orange in *P. abanticus*). A blue spot between the eye and the mouth (vs. absent in *P. abanticus*, in *P. colchicus*, and in *P. strandjae*) and a white spot in front of anal and pectoral fins in males (vs. absent in *P. abanticus* and *P. strandjae*).

Phoxinus kottelati is further distinguished from *P. abanticus* by having a longer and slenderer caudal peduncle (caudal peduncle depth 2.3–2.9 times its length, vs. 1.8–2.3. It further differs from *P. abanticus* by having more lateral line scales (77–90 vs. 60–69). *P. kottelati* is distinguished from *P. abanticus* by the absence of red pigments on dorsal-fin base in live specimens (vs. present). *Phoxinus kottelati* is distinguished from *P. radeki* by having the snout length almost equal to the interorbital distance (vs. snout length greater than interorbital distance) and a deeper caudal peduncle (its depth 9–12% SL mean 10.3 vs. 8–10 mean 9.2). It further differs from *P. radeki* by having shorter and dark-brown rectangular bars along the lateral line (13–16 vs.10–14) and head length 1.1–1.4 mean 1.3 times in body depth (vs. 1.3–1.5 mean 1.4). *P. kottelati* has dark stripes on the middle part of the flank in males, while *P. radeki* has no dark stripes on the middle part of the flank in males

Description. The general appearance is shown in Figs 2–5, and morphometric data are given in Table 1. The largest examined specimen 70 mm SL. The body moderately elongated, dorsal profile and ventral profile slightly more convex than ventral one. The dorsal profile of head markedly more convex than ventral one. The mouth terminal or slightly subterminal, upper lip and snout not projecting

Figure 3. *Phoxinus kottelati*, FFR 2328: holotype, 49 mm SL, male; Türkiye: stream Koca.

Figure 4. *Phoxinus kottelati*, from top, FFR 2323, 57 mm SL, male; 55 mm SL, female Türkiye: stream Akgüney.

Figure 5. *Phoxinus kottelati*, from top: FFR 2310, 48 mm SL female; 49 mm SL male; stream Koca.

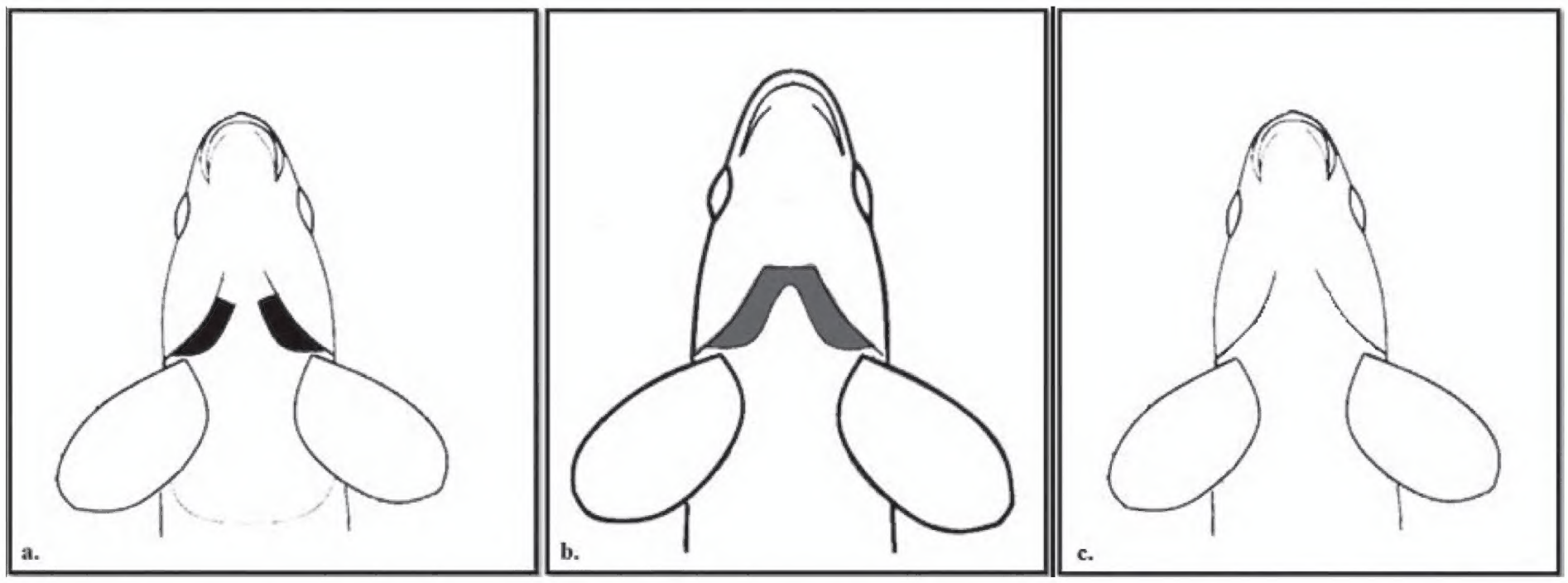

Figure 6. Breast scale shape of **a.** *Phoxinus kottelati* and *P. radeki* **b.** *P. strandjae* **c.** *P. abanticus* (Bayçelebi et al. 2024).

beyond tip of lower jaw. The tip of upper lip at level or above level of lowest point of eye. Snout slightly pointed. The snout short and its upper profile straight or slightly convex. The snout length almost equal to the interorbital distance, its greater than the eye diameter. The caudal peduncle length 2.3–2.9 times in depth of the caudal peduncle.

The lateral line complete, with 77–90 scales, and almost reaching to caudal-fin base; 9–15 scale rows between lateral line and dorsal-fin origin; and 8–10 scale rows between lateral line and anal-fin origin. Dorsal fin with three simple and 7½ branched rays, outer margin straight or slightly convex. Pectoral-fin with 14–18 rays, outer margin convex. Pelvic fin with 8 branched rays, outer margin convex. Anal fin with three simple and 7½ branched rays, outer margin straight to convex. The caudal fin, forked.

Coloration. The specimens were fixed in formalin: back and upper parts of flank brown or grey, the lower part of flank yellowish or light brown, and belly yellowish. There are 13–16 broad and dark-brown rectangular bars along the lateral line. Dorsal, pectoral, anal, and caudal fins grey or light grey (except for some individuals), pelvic fin yellowish. In live male specimens during the spawning period: Z1 greyish with small and irregularly shaped brownish spots; Z2 a light green stripe along the body; Z3 and Z4 almost completely the sides of the body are distinctly green; Z5 a yellowish line from pectoral to anal fins origin and orange between anal- and caudal-fin base; a blue spot between the eye and the mouth; edges of the lips, the base of dorsal, pelvic, and anal fins red. Pelvic and anal fin bases red; a white spot in front of anal and pectoral fins. In females: Z1 and Z2 light grey; Z3 a green stripe along; Z4 and Z5 yellowish; all fins yellowish or greyish. A gery spot between the eye and the mouth (Fig. 2).

Sexual dimorphism. Males with stronger and longer pectoral fins and nuptial tubercles on the head. Additionally, during the breeding season, a number of the body's chromatophores are triggered, giving mature individuals their glossy appearance: Leucophores (white shine), iridophores (iridescent shine), erythrophores (orange/red pigments), xanthophores (yellow pigments), and melanophores (brown/black pigments) (Fuji 2000; Denys et al. 2020) (See coloration and Fig. 2).

Table 1. Morphometric data of *Phoxinus kottelati* (holotype FFR2328 and paratypes FFR 2310 n = 14; FFR2318 n = 15). Mean values are given in parentheses.

	***P. kottelati* n = 29**		
	Uludere and Aşağıçavuşlu streams		
	Black and Marmara Sea basin		
	Range	**H***	**SD**
Standard length (mm)	**43–61**	**49**	
In per cent of standard length			
Head length	24.1–27.2 (25.9)	26.2	0.9
Body depth at dorsal-fin origin	18.1–23.6 (20.6)	20.2	1.4
Caudal peduncle depth	9.3–11.7 (10.3)	9.7	0.7
Head width$_1$ (ant. margin of the eye)	31.2–41.6 (36.8)	38.9	2.8
Head width$_2$ (post. margin of the eye)	47.4–57.4 (52.0)	52.1	2.5
Head width$_3$ (at opercle)	50.5–61.1 (55.0)	56.7	3.0
Head depth$_1$ at the interorbital region	46.4–54.3 (48.8)	47.5	2.0
Head depth$_2$ (at occiput)	59.4–72.6 (65.3)	63.0	3.2
Eye diameter	20.3–29.9 (25.0)	23.1	2.2
Snout length	23.1–32.2 (28.9)	28.4	1.9
Interorbital width	25.0–36.4 (29.1)	29.6	2.4
Snout width at nostrils	28.1–36.2 (32.1)	33.4	2.1
Snout depth at nostrils	27.6–36.3 (31.9)	31.9	1.8
Predorsal length	47.9–57.3 (55.0)	55.3	1.6
Prepelvic length	43.5–48.8 (46.3)	45.1	1.4
Preanal length	60.2–66.1 (63.5)	62.1	1.3
Pectoral-fin origin to anal fin	36.5–43.4 (40.4)	40.4	2.1
Pectoral-fin origin to pelvic fin	20.0–25.8 (22.9)	21.7	1.5
Pelvic-fin origin to anal fin	15.0–20.0 (17.7)	18.3	1.3
Caudal peduncle length	23.0–27.9 (25.8)	24.1	1.2
Dorsal fin height	18.4–23.0 (20.7)	21.8	1.4
Pectoral-fin length	14.6–21.6 (18.4)	21.1	1.9
Pelvic-fin length	12.5–19.1 (14.9)	16.0	1.6
Anal-fin length	16.1–23.1 (19.9)	21.7	1.3
Upper caudal-fin lobe	17.6–24.1 (21.2)	23.0	1.5

*Holotype.

Table 2. Pairwise genetic distances between Turkish minnows under uncorrected p-distance based on cyt *b* sequences.

	P. kottelati	***P. strandjae***	***P. abanticus***	***P. radeki***
P. kottelati				
P. strandjae	0.021			
P. abanticus	0.028	0.034		
P. radeki	0.030	0.031	0.032	
P. strymonicus	0.048	0.048	0.050	0.048

Etymology. The species is named after Maurice Kottelat for his contribution to the knowledge of the ichthyofauna of Euroasia. A noun in genitive, indeclinable.

Distribution. *Phoxinus kottelati* is presently known from the stream Koca, drainage of Lake Manyas, Marmara Sea basin, and the streams Uludere and Akgüney, Black Sea basin (Fig. 1). It occurs in the cold, high-oxygen waters of large lowland rivers and swift-moving mountain streams.

Discussion

Türkiye is situated at the intersection of Central Asia, Africa, the Middle East, and Europe (Şekercioğlu et al. 2011). This unique geographical location is the result of geological processes that have taken place over millions of years (Tavşanoğlu 2016). Geological processes not only positioned Anatolia as a region where species from different biogeographic zones can coexist but also create the necessary potential for topographic diversity and variability through plate tectonic movements (Tavşanoğlu 2016).

The country's mountainous structure, climatic characteristics, valleys, and plains have provided a variety of habitats and local climatic conditions, thereby supporting species survival and contributing to diversification through isolation (Çolak and Rotherham 2006; Şekercioğlu et al. 2011; Tavşanoğlu 2016). Anatolia hosts numerous large-scale topographic barriers that have historically limited the dispersal of species entering the region from different directions. As a result, the distribution of many species have remained restricted to certain regions within Anatolia (Atalay 2006; Tavşanoğlu 2016).

Finally, the distribution pattern of *Phoxinus* species across Türkiye, as illustrated in the present map (Fig. 1), demonstrates a distinct concentration of diversity in the country's northwestern part. This pattern can be explained by a combination of geological history, paleohydrological dynamics, climate, and ecological factors, etc., that have shaped the region's biogeography. Overall, the observed distribution emphasizes the northwestern part of Türkiye as an important biogeographical zone for *Phoxinus*. Further research on ecological niche modelling and fine-scale phylogeography could help to understand the mechanisms supporting this distribution pattern.

This paper, using molecular techniques, is related to the recently described species *Phoxinus kottelati*. Individuals from three different populations were examined: Koca, Uludere, and Akgüney streams from the Marmara and Black Sea basins. The results revealed a reliable genetic distance of this minnow from geographically neighboring clades and species and a certain morphological distinctiveness. The cyt *b* marker was used to aid in species identification. Cyt *b* gene sequences were analyzed in four *Phoxinus* species in Türkiye. As a result of the phylogenetic analysis, *Phoxinus* species in Türkiye were divided into three main clades supported by high bootstrap values. The first clade consisted of *Phoxinus kottelati* sp. nov. and *P. strandjae*, while other clades contained *P. abanticus* and *P. radeki*. *Phoxinus kottelati* sp. new constituted a highly supported clade sister to *P. strandjae* (Fig. 7), and the p-distance in the mitochondrial DNA (mtDNA) cyt *b* gene is 2.1%. Pairwise distances between species ranged from 2.1% (*Phoxinus kottelati* and *P. strandjae*) to 3.4% (*P. abanticus* and *P. strandjae*) (Table 2).

Phoxinus kottelati has a restricted distribution limited to a few streams (Koca, Uludere, and Akgüney); its survival is directly linked to the conservation of these habitats. For fish habitats in general, agricultural runoff, urban development, hydroelectric satellites, etc. pose additional challenges to the water quality and ecological integrity of these streams. Protecting this species requires a comprehensive conservation strategy, including habitat protection, pollution control, and monitoring of invasive species.

Figure 7. The phylogenetic tree generated by using the maximum likelihood method based on the mtDNA cyt *b* (cytochrome b) gene. The bootstrap values are indicated above nodes on the tree if 50% or higher.

Comparative material

Examined materials are already listed by Turan et al. (2023) and Bayçelebi et al. (2024).

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